The contribution of phenolic acids to the anti-inflammatory activity of mushrooms: screening in phenolic extracts, individual parent molecules and synthesized glucuronated and methylated derivatives

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ABSTRACT

In the present study, the ethanolic extracts of fourteen edible mushrooms were investigated for their anti-inflammatory potential in LPS (lipopolysaccharide) activated RAW 264.7 macrophages. Furthermore the extracts were chemically characterized in terms of phenolic acids and related compounds. The identified molecules (*p*-hydroxybenzoic, *p*-coumaric and cinnamic acids) and their glucuronated and methylated derivatives obtained by chemical synthesis were also evaluated for the same bioactivity, in order to establish structure-activity relationships and to comprehend the effects of *in vivo* metabolism reactions in the activity of the compounds. The extracts of *Pleurotus ostreatus, Macrolepiota procera, Boletus impolitus* and *Agaricus bisporus* revealed the strongest anti-inflammatory potential (EC_{50} values 96 ± 1 to 190 ± 6 µg/mL, and also the highest concentration of cinnamic acid (656 to 156 µg/g), which was also the individual compound with the highest anti-inflammatory activity. The derivatives of *p*-coumaric acid revealed the strongest properties, specially the derivative methylated in the carboxylic group (CoA-M1) that exhibited similar activity to the one showed by dexamethaxone used as anti-inflammatory standard; by contrast, the derivatives of *p*-hydroxybenzoic revealed the lowest inhibition of NO production. All in all, whereas the conjugation reactions change the chemical structure of phenolic acids and may increase or decrease their activity, the glucuronated and methylated derivatives of the studied compounds are still displaying anti-inflammatory activity.

Keywords: Edible Mushrooms; Phenolic acids; glucuronated and methylated derivatives; Anti-inflammatory; Nitric oxide production; HPLC-PDA

1. INTRODUCTION

Inflammation is considered to be part of the complex biological response to remove injury or harmful stimuli such as pathogens, damaged cells, or irritation and this is a central feature of many pathophysiological conditions such as atherosclerosis, obesity, metabolic syndrome, diabetes (Pradhan, 2007) and even several types of cancers (Moro et al., 2012).

When cells are exposed to immune stimulants, the pro-inflammatory cells, such as macrophages, monocytes, or other host cells, start to produce cytokines and other mediators, which initiate the inflammation process. Among the various inflammatory mediators, the most common are interleukins (IL-1β, IL-6, IL-8), tumour necrosis factor (TNF-α), nuclear factor-κB (NF-κB), intercellular adhesion molecule-1 (ICAM-1), inducible type cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), 5-lipooxygenase (5-LOX), and inducible nitric oxide synthase (iNOS) that leads to the production of reactive nitrogen species such as nitric oxide (NO). Overproduction of these inflammatory mediators leads to different kinds of cell damage (Kanwar, Kanwar, Burrow, & Baratchi, 2009).

Recently, the nonsteroidal anti-inflammatory drugs (NSAIDs) are usually the most commonly administered drugs to reduce inflammation in the body. Many studies, however, have shown that the long-term administration of NSAIDs has the potential for significant side effects on the gastrointestinal tract with numerous harmful effects such as mucosal lesions, bleeding, peptic ulcers, and intestinal perforation (Dugowson & Gnanashanmugam, 2006). Recent studies show that NSAIDs are also associated with a relatively high incidence of renal adverse drug reactions, nephrotic syndrome, high blood pressure, acute tubular necrosis and cardiovascular toxicity (Elsayed, Hesham, Mohammad, & Ramlan, 2014).

As a result, research studies are now being channelled towards discovery of bioactive compounds with ability to suppress the production of inflammatory mediators. A good model to test potential anti-inflammatory drugs are macrophages, which are large specialized cells

62 that engulf and digest cellular debris, microbes or cancer cells in a process called
63 phagocytosis. They play an important role in non-specific host defence mechanisms and help
64 to initiate other defence mechanisms. Beyond stimulating the immune system, macrophages
65 play a crucial role in the inflammatory response through the release of a variety of factors,
66 such as NO, TNF-α, IL-1β, IL-6, in response to an activating stimulus, e.g.
67 lipopolysaccharide (Moro et al., 2012).
68 Mushrooms are widely appreciated all over the world not only for their culinary and
69 nutritional properties (Kalac, 2009), but also for their pharmacological value as sources of
70 important bioactive molecules, such as antioxidant (Puttaraju, Venkateshaiah, Dharmesh,
71 Urs, & Somasundaram, 2006; Ferreira, Barros, & Abreu, 2009; Heleno, Martins, Queiroz, &
72 Ferreira, 2015), antitumor (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Ferreira, Vaz,
73 Vasconcelos, & Martins, 2010, Carocho & Ferreira, 2013), antimicrobial (Alves, Ferreira,
74 Dias, Teixeira, Martins, & Pintado, 2012; Alves, Ferreira, Froufe, Abreu, Martins, & Pintado,
75 2013), immunomodulator (Borchers, Krishnamurthy, Keen, Meyers, & Gershwin, 2008),
76 antiatherogenic (Mori, Kobayashi, Tomita, Inatomi, & Ikeda, 2008) and hypoglycemic
77 compounds (Hu, Wang, Lien, Liaw, & Lee, 2006). Due to these properties, they have been
78 recognized as functional foods, as well as valuable sources of natural medicines and
79 nutraceuticals (Lindequist, Niedermeyer, Jülich, 2005; Guillamón et al., 2010).
80 Moreover, mushrooms have also demonstrated some anti-inflammatory potential based on
81 their ability to reduce the production of inflammatory mediators (Padilha, Avila, Sousa,
82 Cardoso, Perazzo, & Carvalho, 2009; Elsayed et al., 2014). Previous research studies have
83 been carried out on several mushroom species, mainly in methanolic and ethanolic extracts
84 (**Table 1**). Different compounds have been pointed out as the responsible for the anti-
85 inflammatory activity such as β-glucans (Nosálóva, Bobek, Cerna, Galbavy, & Tvrtina,
86 2001), triterpenes (Ma, Chen, Dong, & Lu, 2013), glycoproteins (Gunawardena et al., 2014)

and even phenolic compounds (Moro et al., 2012). However, not much is known about the phenolic bioactive forms *in vivo*; these compounds are metabolized and circulate in the organism as glucuronated, sulfated and methylated metabolites, displaying higher or lower bioactivity (Heleno et al., 2015).

Therefore, the present study aimed to investigate the anti-inflammatory activity of selected mushroom species from the Northeast of Portugal, using ethanolic extracts in LPS activated RAW 264.7 macrophages. Furthermore, after characterization of the extracts in terms of phenolic acids and related compounds, the identified individual parent molecules and their synthesised glucuronated and methylated derivatives were evaluated for the same bioactivity, in order to establish structure-activity relationships.

2. MATERIALS AND METHODS

2.1. Mushroom species and extracts preparation

Ten wild mushroom species (*Amanita caesaria* (Scop.) Pers., *Boletus aereus* Bull., *B. edulis* Bull., *B. flagrans* Vittad., *B. impolitus* Fr., *B. reticulatus* Schaeff., *Cantharellus cibarius* Fr., *Lactarius deliciosus* (L. ex Fr.) S.F. Gray, *Macrolepiota procera* (Scop.) Singer and *Morchella esculenta* Fr.), collected in the Northeast of Portugal, and four cultivated species (*Agaricus bisporus* (J.E. Lange) Emil J. Imbach, *A. bisporus* Portobello (J.E. Lange) Emil J. Imbach, *Pleurotus eryngii* (DC.) Quél. and *Pleurotus. ostreatus* (Jacq. ex Fr.) P. Kumm.) were used in the present study. All species were deposited in the herbarium of the School of Agriculture in Polytechnic Institute of Bragança, and were previously characterized by the research group in terms of nutritional value and chemical composition (including primary and secondary metabolites) (Barros, Dueñas, Ferreira, Baptista, & Santos-Buelga, 2009, Grangeia, Heleno, Barros, Martins, & Ferreira, 2011; Heleno, Barros, Sousa, Martins, Santos-Buelga, & Ferreira., 2011; Reis et al., 2011; Pereira, Barros, Martins, & Ferreira, 2012; Reis,

Barros, Martins, & Ferreira, 2012; Heleno et al., 2013b). Their antioxidant, antimicrobial and antitumor properties were also previously evaluated by the group (http://esa.ipb.pt/biochemcore/index.php/studied-mushrooms). In the present work, the *in vitro* anti-inflammatory activity was evaluated in ethanolic extracts prepared as follows. Lyophilized (Ly-8-FM-ULE, Snijders, Holland) mushroom powder (20 mesh) of each species (0.5 g) was extracted with ethanol (15 mL), by maceration with stirring for 1 h. Then, the extract was filtered through Whatman no 4 filter paper and the extraction procedure was repeated one more time. The filtrate was rotary evaporated to remove ethanol and the extraction yield was calculated by measuring the extract weight.

2.2. Reagents

Acetonitrile 99.9% was of high-performance liquid chromatography (HPLC) grade from Lab-Scan (Lisbon, Portugal). *p*-Hydroxybenzoic acid, *p*-coumaric acid, cinnamic acid, Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, Griess reagent system (Promega), DMSO, sulphorodamine B (SRB) and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA). All other chemicals and solvents were of analytical grade and purchased from common suppliers.

2.3. Chemical characterization of the extracts

The dry mass of each mushroom extract was re-dissolved in water/ethanol (50:50, *v/v*) and filtered through a 0.22 µm nylon disposable filter for HPLC analysis. The analysis was performed using a Shimadzu 20A series ultra-fast liquid chromatograph (UFLC, Shimadzu Coperation, Kyoto, Japan). Separation was achieved on a Waters Spherisorb S3 ODS2 C_{18} column (3 µm, 150 mm x 4.6 mm) column thermostatted at 35 ºC. The solvents used were:

(A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was: 10% B to 15% B over 5 min, 15–25% B over 5 min, 25–35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Detection was carried out in a photodiode array detector (PDA), using 280 nm as the preferred wavelength. The phenolic acids (group of phenolic compounds identified in the samples) were quantified by comparison of the area of their peaks recorded at 280 nm with calibration curves obtained from commercial standards of each compound: protocatechuic acid (y = 164741x, R^2=0.999), *p*-hydroxybenzoic acid (y = 113523x, R^2=0.999), *p*-coumaric acid (y = 433521x, R^2=0998) and cinnamic acid (y = 583527x, R^2=0.998), 5 to 80 µg/mL. The results were expressed as µg per g of extract.

2.4. Phenolic acids and synthesised derivatives

p-Hydroxybenzoic acid, *p*-coumaric acid and cinnamic acid, identified in the mushroom extracts, were used as staring reagents for the synthesis of methylated and glucuronated derivatives of the identified compounds (**Figure 1**); these compounds were previously synthesized and completely characterized by the authors (Heleno et al., 2013a; Heleno et al., 2014b). Briefly, the glucuronated derivatives (HA-GP, CoA-GP and CA-GP) were obtained by reacting the parent molecules with acetobromo-α-*D*-glucuronic acid methyl ester under argon and using DMSO (dimethylsulfoxide) as solvent at room temperature. The methylated derivatives (HA-M1, CoA-M1 and CA-M) were prepared using methanol and sulphuric acid at room temperature; HA-M2 and CoA-M2 were synthesised using dimethyl sulphate in acetone at room temperature. Finally, HA-M3 and CoA-M3 were obtained by the hydrolysis of compounds HA-M2 and CoA-M2 using ethanol at 65ºC and adjusting the pH to 3. All the synthesised compounds were fully characterized by ^{1}H NMR, ^{13}C NMR, HRMS (high resolution mass spectrometry) and melting point.

2.5. Evaluation of the anti-inflammatory activity

2.5.1. Cells treatment

The anti-inflammatory activity was carried out according to Moro et al. (2012) and García-Lafuente et al. (2014) with some modifications. The mouse macrophage-like cell line RAW 264.7 was cultured in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum, glutamine and antibiotics at 37 °C under 5% CO_2, in humidified air. For each experiment, cells were detached with a cell scraper. A cell density of 5 x 10^5 cells/mL was used, and the proportion of dead cells was less than 5%, according to Trypan blue dye exclusion test.

Cells were seeded in 96-well plates at 150,000 cells/well and allowed do attach to the plate overnight. Then, cells were treated with the different concentrations of each one of the extracts for 1h. Dexamethasone (50 µM) was used as a positive control for the experiment. The following step was the stimulation with LPS (1 µg/mL) for 18h. The effect of all the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in nitric oxide (NO) basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in supplemented DMEM.

2.5.2. Nitric oxide determination

Both the extracts, and the pure identified compounds and their synthezised derivatives were submitted to the anti-inflammatory activity assay. The ethanolic extracts were dissolved in water (non cytotoxic solvent) at 8 mg/mL, while the identified individual compounds and their synthesised methylated and glucuronated derivatives were dissolved in DMSO at 50% concentration in stock solutions. These solutions were then submitted to further dilutions (400 µg/mL to 50 µg/mL and 2500 µM to 39 µM, for the extracts and compounds,

187 respectively) in order to determine effective concentrations (Moro et al., 2012; García-
188 Lafuente et al., 2014).
189 For the determination of nitric oxide, Griess Reagent System kit was used, which contains
190 sulphanilamide, N-(1-napthyl)ethylenediamine hydrochloride (NED) and nitrite solutions. A
191 reference curve of the nitrite (sodium nitrite 100 µM to 1.6 µM; y=0.0066x+0.1349;
192 R^2=0.9986) was prepared in a 96-well plate. The cell culture supernatant (100 µL) was
193 transferred to the plate and mixed with Sulphanilamide and NED solutions, 5-10 minutes
194 each, at room temperature. The nitric oxide produced was determined by measuring the
195 absorbance at 540 nm (microplate reader ELX800 Biotek), and by comparison with the
196 standard calibration curve.
197
198 **2.6. Statistical analysis**
199 For all the experiments three samples were analyzed and all the assays were carried out in
200 triplicate. The results are expressed as mean values ± standard deviation (SD). The
201 differences between the different samples were analyzed using one-way analysis of variance
202 (ANOVA) followed by Tukey's honestly significant difference post hoc test with α = 0.05,
203 coupled with Welch's statistic. This treatment was carried out using SPSS v. 22.0 program.
204
205 **3. RESULTS AND DISCUSSION**

206 ***3.1. Chemical characterization of the extracts***
207 Ethanolic extracts were prepared from fourteen different edible mushroom species: *Agaricus*
208 *bisporus, A. bisporus* Portobello*, Amanita caesaria, Boletus aereus, B. edulis, B. flagrans*, *B.*
209 *impolitus*, *B. reticulatus*, *Cantharellus cibarius*, *Lactarius deliciosus, Macrolepiota procera,*
210 *Pleurotus eryngii*, Pleurotus *ostreatus* and *Morchella esculenta*. One phenolic acid (*p*-
211 hydroxybenzoic acid) and two cinnamic acids (*p*-coumaric and cinnamic acids) were detected
212 by HPLC-PDA in the different extracts; their concentrations are presented in **Table 2**. The

phenolic composition of the mushrooms seems to be characterised by the presence of phenolic acids, being cinnamic acid the major compound in most cases. No peaks were found in the extracts whose UV spectra could be associated to hydroxycinnamic acids or their tartaric or quinic esters, nor were flavonoids found. The fact that no flavonoids were identified is not unexpected since, it is assumed that only plants possess the biosynthetic ability to produce flavonoids and not animals and fungi (Iwashina, 2000). Furthermore, these compounds have been extensively reported in mushroom species (Barros et al., 2009; Vaz et al., 2011).

The three compounds were only identified and quantified in three of the analysed species, *B. aereus*, *B. impolitus* and *P. ostreatus*, while *A. caesarea*, *C. cibarius* and *L. deliciosus* presented *p*-hydroxybenzoic and cinnamic acids. The other mushroom samples only revealed the presence of cinnamic acid. *P. ostreatus* revealed the highest concentration of phenolic acids mainly due to the high contribution of cinnamic (619±3 µg/g) and *p*-hydroxybenzoic (295±5 µg/g) acids. *M. procera* and *B. impolitus* also presented high amounts of cinnamic acid (522 ± 1 µg/g and 505 ± 12 µg/g, respectively). The samples *A. bisporus* Portobello, *B. edulis* and *B. flagrans* revealed only the presence of cinnamic acid, but in very low concentration. Reis et al. (2011) and Heleno et al. (2013b) also reported the presence of protocatechuic acid in *A. caesaria* and *M. esculenta*, respectively, although they performed an extraction with methanol:water, while in the present study ethanolic extracts were prepared. Therefore, the different extraction conditions could be responsible for the dissimilarity observed in the phenolic profiles.

3.2. In vitro anti-inflammatory activity of the extracts

The effects of the ethanolic extracts on the production of inflammatory mediators (NO) in RAW 264.7 macrophages, upon stimulation with LPS, are shown in **Figure 2**. The results

238 indicate that most of the assayed extracts inhibited LPS-induced NO production in a dose-
239 dependent manner. For an easier comparison of the results, EC_{50} values were calculated
240 based on 50% of inhibition of NO production (**Table 3**). The most efficient species was *P.*
241 *ostreatus* (96 ± 1 µg/mL), followed by *M. procera* (162 ± 2 µg/mL), *B. impolitus* (166 ± 10
242 µg/mL) and *A. bisporus* (190 ± 6 µg/mL). These results are in agreement with the reports by
243 Moro et al. (2012) and Gunawardena et al. (2014) that described anti-inflammatory activity,
244 by decreasing NO levels in RAW 264.7 cells, of ethanolic and methanolic extracts of *A.*
245 *bisporus*, *C. cibarius*, *L. deliciosus* and *P. ostreatus* (**Table 1**). The ethanolic extracts from *A.*
246 *bisporus* Portobello, *B. edulis* and *B. flagrans* appeared as the less active, showing EC_{50}
247 values above 400 µg/mL. Moro et al. (2012) reported some activity in the case of methanolic
248 extracts of *B. edulis* with 10% inhibition of NO production at concentrations of 500 µg/mL.
249 Although many substances may participate in the anti-inflammatory activity, phenolic
250 compounds have been largely recognised as natural molecules with anti-inflammatory
251 effects. Positive correlations have been found between phenolic compounds and anti-
252 inflammatory effects (Cheung, Cheung, & Ooi, 2003; Kim et al., 2008). In the present study,
253 it was also observed that the extract with the highest anti-inflammatory activity showed the
254 highest levels of cinnamic and phenolic acids.
255
256 ***3.3. In vitro anti-inflammatory activity of the compounds identified in the extracts***
257 To demonstrate this supposition, the activity of the individual compounds present in the
258 extracts were further evaluated (**Table 4**). Phenolic acids, and cinnamic acid, that are the
259 main responsible molecules for the bioactivities exhibited by mushrooms are well known for
260 their biological properties, mainly due to the OH groups present in their chemical structure,
261 either in the carboxylic group or in the phenolic ring (Heleno et al., 2015). Cinnamic acid
262 (CA) showed the highest anti-inflammatory activity presenting the lowest EC_{50} values (182 ±

16 µM), followed by *p*-hydroxybenzoic (239 ± 29 µM) and *p*-coumaric (442 ± 33 µM) acids. Cinnamic acid presents a carboxylic group and no OH groups in the benzene ring. *p*-Hydroxybenzoic and *p*-coumaric acids present an OH group in the *para* position that is usually a position described as having biological properties. However, in the present study and comparing the activity exhibited by these three molecules, the OH group in the *para* position seems to decrease the anti-inflammatory ability, maybe due to the mechanism of action in this specific cell line. Several authors suggest that the anti-inflammatory activity is related with the ability of the compounds to inhibit the activity of the cyclooxygenase (COX) enzyme, which is responsible for the synthesis of prostaglandins, mediators with a great ability to induce inflammation (Lee et al., 2006; Tanaka et al., 2009). Studies on the mechanism of action of these molecules are important to better understand their behaviour. Nevertheless, these results are in agreement with the ones reported by our research group for the antimicrobial activity of these compounds, where cinnamic acid also revealed the strongest activity followed by *p*-hydroxybenzoic and *p*-coumaric acids (Heleno et al., 2014a).

3.4. In vitro anti-inflammatory activity of glucuronated and methylated derivatives of the identified compounds

As phenolic acids are metabolized in the organism and suffer conjugation reactions originating different metabolites such as glucuronated and methylated derivatives, a change in their effects or activity may also occur. Thus, the bioactivity of the parent molecule can be increased, decreased or maintained (Heleno et al., 2015). Hereby the glucuronated and methylated derivatives of the considered acids were analysed and compared to the one of the parent molecule. Among the glucuronated derivatives, CoA-GP (glucuronated derivative of *p*-coumaric acid) presented strong anti-inflammatory activity (58 ± 5 µM), being comparable to the activity of the standard dexamethaxone (40 ± 4 µM), followed by the glucuronated

288 derivatives of cinnamic (CA-GP) (179 ± 71 μM) and *p*-hydroxybenzoic (HA-GP) (1901 ±
289 104 μM) acids. Contrarily to the results verified for the anti-inflammatory activity of the
290 three parental molecules, the glucuronated derivative of *p*-coumaric acid exhibited the
291 strongest activity, presenting an acetylated glucuronide in the carboxylic group and an OH
292 group in the *para* position equal to the glucuronated derivative of *p*-hydroxybenzoic acid,
293 but, with a double bond in the middle increasing the lateral chain. Thus, the strongest activity
294 can be related with the double bound that stabilizes the molecule and the reactivity of the
295 benzenic ring to be able to inhibit the activity of COX. Regarding the methylated derivatives,
296 those of *p*-coumaric acid presented higher activity than the ones of cinnamic or *p*-
297 hydroxybenzoic acids; in particular, the methylated derivative CoA-M1, with an ester instead
298 of the carboxylic group, revealed very strong activity (35 ± 2 μM), very close to the
299 dexamethaxone value (40 ± 4 μM). Also in the case of methylated derivatives it seems that
300 the double bound of *p*-coumaric acid allows a higher ability to interact with the tested cell
301 line, while in the case of *p*-hydroxybenzoic acid that has no double bound, the ester and OH
302 groups are more close to each other and can chemically interact changing the molecule
303 stability and decreasing the bioactivity.
304 Comparing the activity of each parent molecule and the corresponding glucuronated and
305 methylated derivatives, the order was as follows: *p*-hydroxybenzoic acid: HA > HA-M3 >
306 HA-M2 >HA-M1 > HA-GP; *p*-coumaric acid: CoA-M1 > CoA-GP > CoA-M2 > CoA-M3 >
307 CoA; and cinnamic acid: CA-GP > CA > CA-M. *p*-Hydroxybenzoic acid showed higher
308 activity than the corresponding derivatives, with HA-M3 as the most active compound; the
309 decreased anti-inflammatory activity observed for HA-M1, HA-M2 and HA-GP could be
310 explained by the esterification of the carboxylic group. On the contrary, all *p*-coumaric acid
311 derivatives showed higher activity than the parent molecule (CoA), particularly the
312 methylated compound CoA-M1, suggesting that in this case the esterification of the

carboxylic group together with the free OH group in *para* position increased their anti-inflammatory activity. The glucuronated derivative of cinnamic acid (CA-GP) maintained its activity in comparison with the parent molecule (CA), while the methylated one (CA-M) decreased it.

These results are in relatively good agreement with the ones previously reported by our research group comparing the antimicrobial activity of *p*-hydroxybenzoic, *p*-coumaric and cinnamic acids and their respective methylated and glucuronated derivatives. The methylation of *p*-hydroxybenzoic and cinnamic acids decreased the antimicrobial activity, while the methylation of *p*-coumaric acid increased it (Heleno et al., 2014a); moreover, the glucuronidation of the parent molecules also decreased the antimicrobial activity, except for HA-GP that showed higher antifungal activity against some pathogenic strains (Heleno et al., 2013a).

4. Conclusion

Overall, the mushroom species: *P. ostreatus, M. procera, B. impolitus* and *A. bisporus* revealed the strongest anti-inflammatory potential presenting the highest inhibition of NO production. These mushroom species also revealed the highest concentration in cinnamic acid, which was also the individual compound that presented the strongest anti-inflammatory activity and, therefore, could play an important role in the observed activity. However, the conjugation reactions occurring in the organism can change the chemical structure of cinnamic and phenolic acids increasing or decreasing their *in vivo* anti-inflammatory activity. The possible metabolites previously synthesised by the authors and tested in the present work are still displaying activity, in some cases like CoA-GP and CoA-M1 higher than the parent compound and very close to the activity exhibited by dexamethaxone used as anti-inflammatory standard.

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Table 1. Some previous studies on anti-inflammatory activity of different mushroom species evaluated through NO assay.

Species	Country	Extract	Inhibition of NO production	References
Agaricus bisporus	Spain	Methanol	30% at 0.5 mg/mL	Moro et al., 2012
	Australia	Ethanol	50% at 0.032 mg/mL	Gunawardena et al., 2014
Boletus edulis	Spain	Methanol	10% at 0.5 mg/mL	Moro et al., 2012
Cantharellus cibarius	Spain	Methanol	70% at 0.5 mg/mL	Moro et al., 2012
Caripia montagnei	Brazil	Acetone 80%, methanol	43% at 10 mg/kg 54% at 30 mg/kg 49% at 50 mg/kg	Queiroz et al., 2010
Cratarellus cornucopoides	Spain	Methanol	55% at 0.5 mg/mL	Moro et al., 2012
Flammulina velutipes	Australia	Ethanol	50% at 0.024 mg/mL	Gunawardena et al., 2014
Inonotus obliquus	China South Korea	Ethanol Ethanol	65% at 40 µg/mL 50% at 89 µg/mL	Ma et al., 2013 Park et al., 2005
Lactarius deliciosus	Spain	Methanol	40% at 0.5 mg/mL	Moro et al., 2012
Lentinus edodes	Australia	Ethanol	50% at 0.027 mg/mL	Gunawardena et al., 2014
Pleurotus ostreatus	Australia Spain	Ethanol Methanol	50% at 0.077 mg/mL 15% at 0.5 mg/mL	Gunawardena et al., 2014 Moro et al., 2012
Pleurotus tuber-regium	Belgium	Ethanol	70% at 0.5 mg/mL	Liu et al., 2014
Tricholoma matsutake	South Korea	Dichloromethane	47% at 2 mg/mL	Lim et al., 2007

Table 2. Cinnamic and phenolic acids identified and quantified by HPLC-PDA in the ethanolic extracts of the analysed mushrooms.

Mushroom species	Extraction yield (%)	*p*-Hydroxybenzoic acid (µg/g)	*p*-Coumaric acid (µg/g)	Cinnamic acid (µg/g)
Agaricus bisporus	6.9 ± 0.7^{abcd}	nd	nd	149 ± 2^{d}
Agaricus bisporus Portobello	6.0 ± 0.1^{bcd}	nd	nd	11 ± 1^{hi}
Amanita caesaria	5.4 ± 0.5^{cd}	57 ± 3^{e}	nd	156 ± 3^{d}
Boletus aereus	7.7 ± 0.6^{abc}	43 ± 1^{f}	74 ± 1^{b}	50 ± 3^{f}
Boletus edulis	10.4 ± 0.5^{a}	nd	nd	14.2 ± 0.4^{gh}
Boletus flagrans	4.9 ± 0.3^{cd}	nd	nd	6.1 ± 0.3^{i}
Boletus impolitus	4.1 ± 0.5^{d}	125 ± 9^{c}	45 ± 2^{c}	505 ± 12^{c}
Boletus reticulatus	9.5 ± 0.3^{ab}	nd	nd	20.3 ± 0.1^{g}
Cantharellus cibarius	4.0 ± 0.3^{d}	151 ± 2^{b}	nd	71 ± 1^{e}
Lactarius deliciosus	3.7 ± 0.4^{d}	108 ± 5^{d}	nd	67 ± 1^{e}
Morchella esculenta	4.0 ± 0.8^{d}	nd	nd	71 ± 3^{e}
Macrolepiota procera	3.7 ± 0.4^{d}	nd	nd	522 ± 1^{b}
Pleurotus eryngii	10.1 ± 0.6^{a}	nd	nd	16 ± 1^{gh}
Pleurotus ostreatus	4.0 ± 0.6^{d}	297 ± 5^{a}	171 ± 1^{a}	619 ± 3^{a}

nd- not detected. ANOVA In each column, different letters mean statistical significant differences ($p<0.05$) between samples.

Table 3. Extract concentrations responsible for 50% of reduction of NO production (EC_{50} values) in RAW 264.7 cell line.

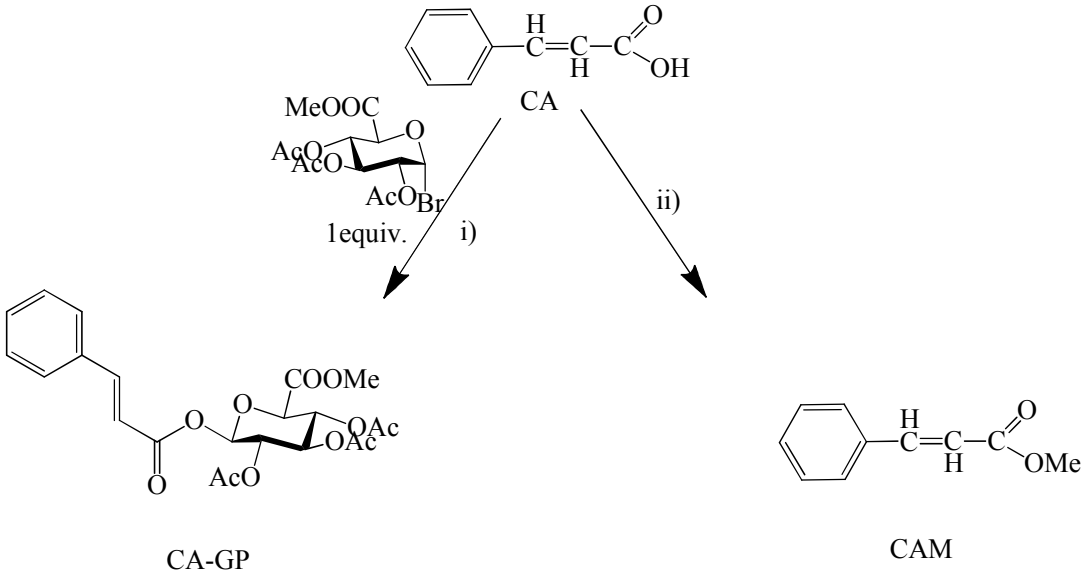
Mushroom species	EC_{50} values (µg/mL)	Mushroom species	EC_{50} values (µg/mL)
Agaricus bisporus	190 ± 6^{ef}	*Boletus reticulatus*	378 ± 28^{ab}
Agaricus bisporus portobelo	$>400^{a}$	*Cantharellus cibarius*	202 ± 17^{e}
Amanita caesaria	186 ± 7^{ef}	*Lactarius deliciosus*	253 ± 14^{d}
Boletus aereus	357 ± 3^{b}	*Macrolepiota procera*	162 ± 2^{g}
Boletus edulis	$>400^{a}$	*Morchella esculenta*	287 ± 9^{c}
Boletus flagrans	$>400^{a}$	*Pleurotus eryngii*	388 ± 17^{a}
Boletus impolitus	166 ± 10^{fg}	*Pleurotus ostreatus*	96 ± 1^{h}

EC_{50} values correspond to 50% of inhibition of the NO production in comparison with the negative control (100% of NO production). In the columns, different letters mean statistical significant differences ($p<0.05$) between samples. Dexamethaxone EC_{50} value = 16 ± 2 µg/mL.

Table 4. Concentrations of the studied acids and their glucuronated and methylated derivatives responsible for 50% of reduction of NO production (EC_{50} values, μM) in RAW 264.7 cell line.

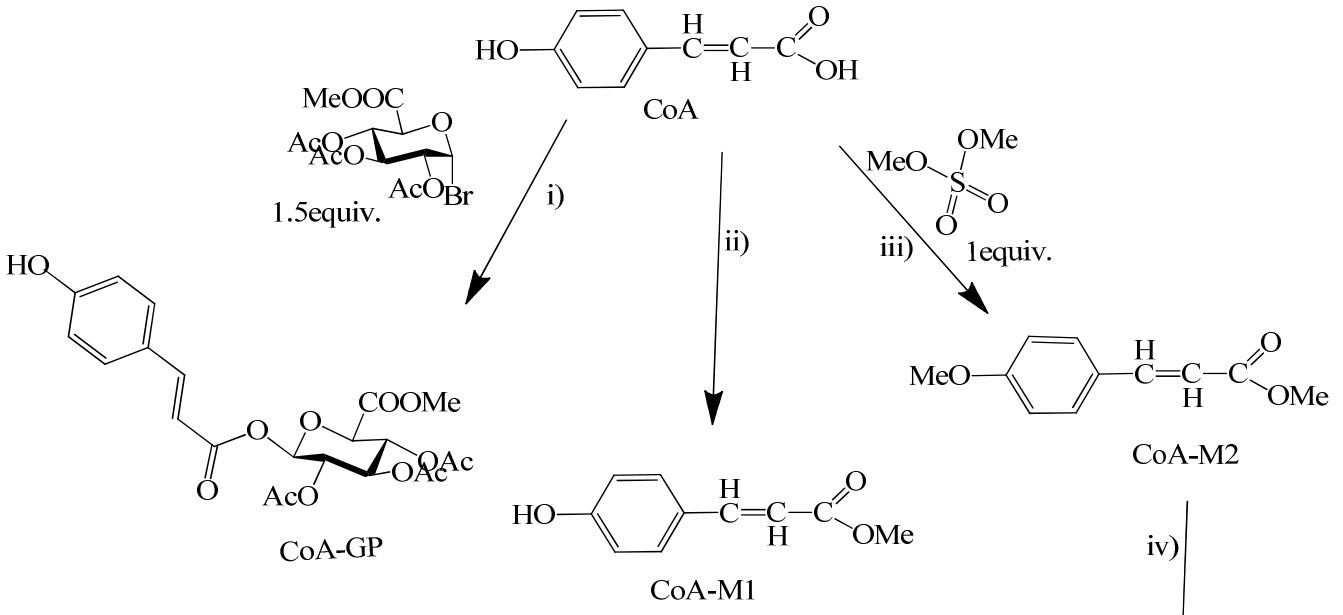
p-Hydroxybenzoic acid and derivatives		*p*-Coumaric acid and derivatives		Cinnamic acid and derivatives	
HA	239 ± 29^{c}	CoA	442 ± 33^{a}	CA	182 ± 16^{b}
HA-GP	1901 ± 104^{a}	CoA-GP	58 ± 5^{c}	CA-GP	179 ± 17^{b}
HA-M1	1825 ± 120^{a}	CoA-M1	35 ± 2^{c}	CA-M	224 ± 16^{a}
HA-M2	526 ± 26^{b}	CoA-M2	128 ± 10^{b}		
HA-M3	509 ± 47^{b}	CoA-M3	129 ± 6^{b}		

EC_{50} values correspond to 50% of inhibition of the NO production in comparison with the negative control (100% of NO production). In each column, different letters mean statistical significant differences ($p<0.05$) between compounds. Dexamethaxone EC_{50} value = 40 ± 4 μM.



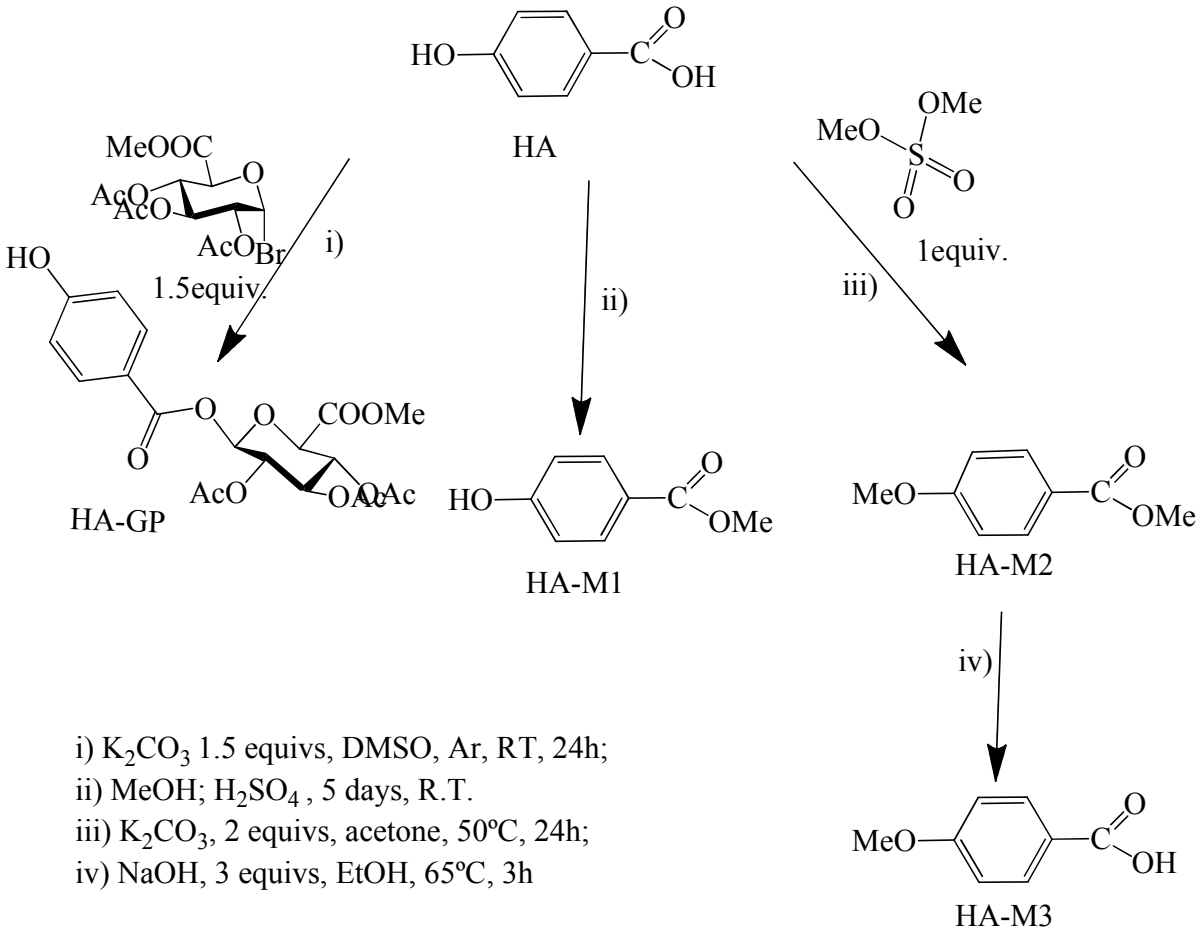
i) K_2CO_3 1.5 equivs, DMSO, Ar, RT, 24h;
ii) MeOH; H_2SO_4 , 5 days, R.T.

A



i) K_2CO_3 1.5 equivs, DMSO, Ar, RT, 24h;
ii) MeOH; H_2SO_4 , 5 days, R.T.
iii) K_2CO_3, 2 equivs, acetone, 50ºC, 24h;
iv) NaOH, 3 equivs, EtOH, 65ºC, 3h

B



C

Figure 1. Synthesis of methylated and glucuronated derivatives of *p*-hydroxybenzoic, *p*-coumaric and cinnamic acids. **A**) i) Glucuronidation of cinnamic acid (CA). CA-GP- cinnamic acid glucuronide protected form, 2,3,4-tri-*O*-acetyl-1-cinnamoyl-D-glucuronic acid methyl ester (Heleno et al., 2013a); ii) Methylation of CA. CAM- methyl 3-phenylacrylate (Heleno et al., 2014b). **B)** i) Glucuronidation of *p*-coumaric acid (CoA). CoA-GP- *p*-Coumaric acid glucuronide protected form, 2,3,4-tri-*O*-acetyl-1-*p*-coumaroyl-D-glucuronic acid methyl ester (Heleno et al., 2014b); ii)-iv) Methylations of CoA. CoA-M1- 3-(4-hydroxyphenyl) acrylate, CoA-M2- methyl-(4-methoxyphenyl) acrylate, CoA-M3- 3-(4-methoxyphenyl) acrylic acid (Heleno et al. 2014b). **C)** i) Glucuronidation of *p*-hydroxybenzoic acid (HA). HA-GP- *p*-hydroxybenzoic acid protected form, 2,3,4-tri-*O*-acetyl-1-*p*-hydroxybenzoyl-D-glucuronic acid methyl ester (Heleno et al., 2013a); ii-iv) Methylations of *p*-hydroxybenzoic acid, HA-M1- methyl 4-hydroxybenzoate, HA-M2- methyl-*p*-anisate, HA-M3- 4-methoxybenzoic acid (Heleno et al., 2014b).

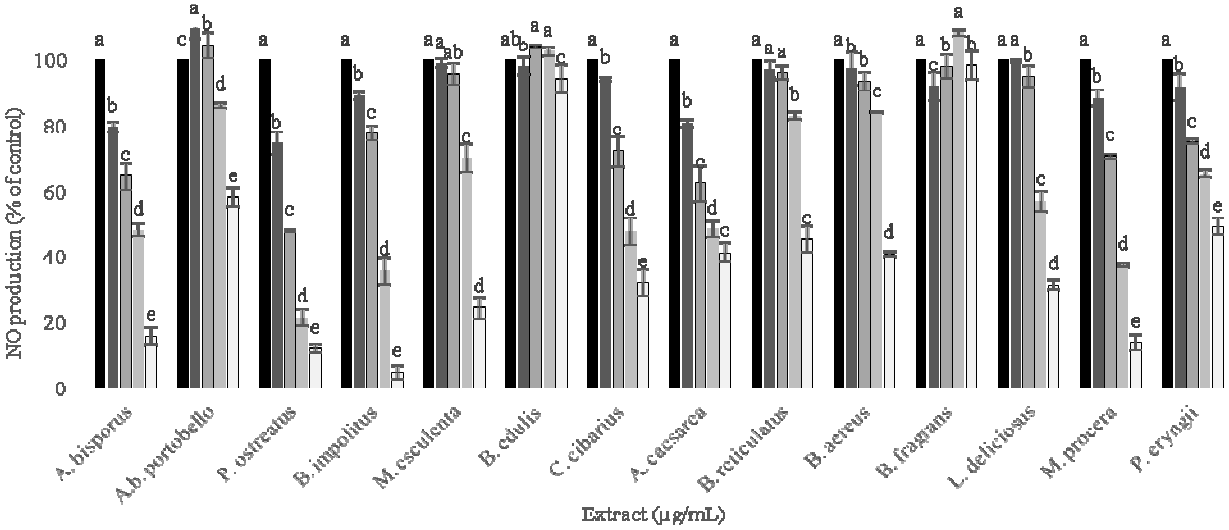


Figure 2. Effects of the ethanolic mushroom extracts in the NO produced by RAW 264.7 cell line. Mean values and standard errors, expressed in relation to the negative control (without extract)- 100% of production. ■ 0 µg/mL; ■ 50 µg/mL; ■ 100 µg/mL; ■ 200 µg/mL; □ 400 µg/mL.